CELLULOSE HYDROLYSIS AND INTEGRATED PROCESSING RESEARCH

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Abstract

A kinetic model for cellulose hydrolysis was proposed that differs from previous models in distinguishing between the adsorption of β-glucosidase and that of CBH and EG enzymes. The model also incorporates inhibition by xylose, which has not been considered previously. Xylose is a major sugar in hydrolyzates of dilute-acid pretreated biomass. The model performed well in predicting cellulose hydrolysis trends at experimental conditions both inside and outside the design space used for parameters estimation, but it could be improved upon by incorporating the phenomenon of enzyme inactivation and differential hydrolysis potential.

Previous corn-stover saccharification work has been carried out with washed pretreated corn stover. We have made some progress in characterizing enzymatic cellulose saccharification under process-relevant conditions and understanding how to configure the overall process to maximize intermediate sugars production. Using pretreated corn stover, the effect of hydrolyzate levels on hydrolysis was studied. Different levels of hydrolyzates exhibited fairly similar glucan conversion using Spezyme, whereas CPN's performance dropped significantly at high hydrolyzate levels indicating susceptibility to increased levels of hydrolyzate toxins. Thus, Spezyme seemed to be less sensitive to hydrolyzate levels than CPN. Another observation from this study is that final cellobiose levels rise with increasing hydrolyzate levels, implying a hampering of β-glucosidase performance for both enzyme preparations. Hence, resistance to cellobiose inhibition is a desirable trait for the next generation of cellulases.

Recommendations for future work are as follows:

- Incorporate enzyme inactivation and hydrolysis capacity factor in the kinetic model
- Evaluate 2nd generation cellulase enzyme preps
- Obtain preliminary performance and mass balance closure information on process-relevant hydrolysate conditioning
- Develop core knowledge on separation processes
- Develop core knowledge on cellulose saccharification in the context of an integrated process
- Explore reactor designs for effectively mixing corn stover slurries

INTRODUCTION

Goals and Objectives

The objective of cellulose hydrolysis effort is to develop and demonstrate integrated enzyme-based cellulose hydrolysis and to improve the understanding of cellulose hydrolysis via kinetic modeling and experimental investigation. In the large context of process technology, a future goal is to demonstrate the integration of core technologies—pretreatment, saccharification, and fermentation (would vary with desired product/s)—using corn stover as a model feedstock and to generate high-quality performance data and mass balances for integrated systems operated under realistic conditions. Fermentation is included only to demonstrate the integrated process and would be product specific. Under contracts with the U.S. Department of Energy (DOE), Genencor International and Novozymes Biotech Inc. are working to reduce enzyme costs by an order of magnitude (Canning 2002, Lamb 2001, Russo 2001). This is a major effort critical to project success.

Background

Enzymatic Hydrolysis

Economical bioconversion requires the following: a well-pretreated substrate, an efficient cellulase system, and an effective microorganism. Cellulose hydrolysis is effected via a synergistic action of endoglucanases, exoglucanases, and β-glucosidases (Figure 1). As a consequence of the action of the first two enzymes, cellobiose and glucose are produced. Both sugars cause end-product inhibition, however, cellobiose is a much stronger inhibitor than glucose. Hence, overcoming this inhibition is one of the challenges in non-SSF (SSF: simultaneous saccharification and fermentation) modes. Table 1, adapted and extended from Esteghlalian et al. (2000), lists enzyme-, substrate- and process-related factors that affect the efficiency of enzymatic hydrolysis, which constitutes a major cost for the overall bioconversion process (Gregg et al 1998).

Process Configurations

The production of ethanol from biomass requires the following basic steps: pretreatment to hydrolyze the hemicellulose, hydrolysis of cellulose to produce glucose, fermentation of sugars to end product, and product recovery. Ethanol is used here as an example product. There are different process configurations, both enzyme based and non-enzyme based that can be used to achieve the overall goal. In the non-enzyme based approach, acid is used for both hemicellulose and cellulose hydrolysis, and the mode is separate saccharification and fermentation (SHF). In the enzymatic approach, SHF, SSF, or simultaneous saccharification and cofermentation (SSCF) can be used. SSCF is a variant of SSF, IN which cofermentation refers to the fermentation of both six-carbon (hexoses, i.e., glucose, mannose, and galactose) and five-carbon (pentoses, i.e., xylose and arabinose) sugars to ethanol.

Although the hydrolysis characteristics of cellulase enzymes to be used in the process are unknown, it is expected that the next generation of cellulases now under development will possess higher specific activities and greater thermostablity than current preparations, but will still be partially subject to inhibition by sugars. As a consequence, a hybrid hydrolysis and fermentation (HHF) process configuration is well suited. In an HHF mode, partial

saccharification is achieved at the optimum temperature for the enzyme, which maximizes enzymatic reaction rates. After achieving a certain degree of saccharification, the reaction mixture is cooled to fermentation temperature, the reactor is inoculated and then operated in an SSF or SSCF mode.

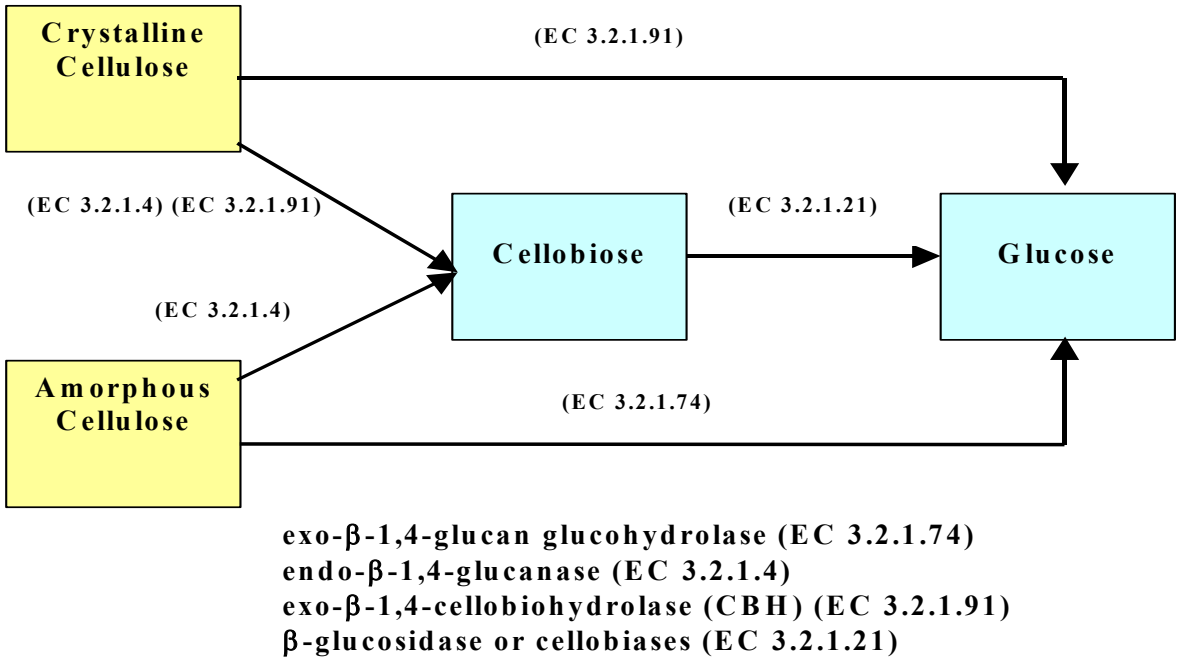

Figure 1. Schematic representation of enzymatic hydrolysis of cellulose.

Table 1. Factors affecting enzymatic hydrolysis

Enzyme Related Factors	Substrate Related Factors	Process Related Factors
Reaction heterogeneity (soluble enzyme vs. insoluble substrate)	Cellulose crystallinity	Process configuration (reconciliation of optimal conditions for cellulases vs. ethanologen)
Irreversible binding of enzymes onto lignin	Cellulose degree of polymerization	Operating parameters: pH, temperature, duration
Gradual loss of synergism in cellulase mixture	Feedstock particle size	Enzyme loading
Substrate dependence of synergism and binding (specificity) of enzyme components	Lignin barrier (content and distribution)	Solids level
End-product inhibition	Substrate's available surface area (pore volume)	Bulk mixing, diffusion limitations
Thermal inactivation of enzyme	Cell wall thickness (coarseness)	Batch vs. continuous mode
	Substrate composition	Reactor design
	Composition and fine structure	

Process Implications of Enzyme Characteristics

Enzymes exhibiting reduced end-product inhibition would favor an SHF type process configuration and the biorefinery concept. *Trichoderma*-based acid cellulases with pH optima of 5.0-5.5 are current candidates for a bioethanol process; however, enzymes with higher pH optima may be more suitable to some situations. Neutral cellulases with pH optima 6.5-7.0, currently used in applications such as stone washing of denim, possess higher EG activity than acid cellulases, but their efficacy for biomass hydrolysis is not known. If thermotolerant neutral cellulases could be developed, these new enzymes would render high-temperature SSF a possibility using a thermophilic microbe such as *Bacillus stearothermophilus*.

Challenges and Knowledge Gaps

The knowledge gaps in this area are: 1) specific process-relevant information on corn stover hydrolysis; although some information is available (Elshafei et al 1991, Kaar & Holtzapple 2000, Wilke et al 1981); 2) optimal conditions for potentially available cellulases vs. ethanologens; and 3) feasibility of other process configurations.

The challenges in this area are: 1) practical and economical ways to overcome end-product inhibition; 2) reactor design for operating at high solids levels; 3) hitherto unknown interactions among unit operations; 4) proper matching of flow rates and equipment volumes for actual demonstration; 5) operational issues if some sections are batch and others continuous; and 6) demonstration of robustness and scalability of the process in an integrated fashion.

RECENT EXPERIMENTAL WORK

This section discusses recent experimental work in the areas of kinetic modeling and cellulase adsorption to improve our understanding of enzymatic hydrolysis, and in process-relevant lignocellulose saccharification and fermentation addressing system performance under realistic conditions.

Kinetic Modeling

The development of a kinetic model, based on observable, macroscopic properties of the overall system, is helpful in the design and economic evaluation of processes for sugar conversion and ethanol production. Kinetic modeling of enzymatic hydrolysis is complicated by the heterogeneous nature of the substrate and multiple enzyme activities. A rigorous model needs to include the phenomena of enzyme adsorption, inactivation, and inhibition.

A multi-reaction kinetic model was developed incorporating these features for closed-system enzymatic hydrolysis of lignocellulosic biomass such as corn stover. Three hydrolysis reactions were modeled, two heterogeneous reactions for cellulose breakdown to cellobiose and glucose, and one homogeneous reaction for hydrolyzing cellobiose to glucose. Cellulase adsorption on to pretreated lignocellulose was modeled via a Langmuir-type isotherm. A competitive mode was used to describe inhibition caused by sugars produced from cellulose, i.e., cellobiose and glucose as well as by xylose, the dominant sugar prevalent in most hemicellulose hydrolyzates;

previously published models have ignored xylose inhibition. The proposed kinetic model is described in the equations below.

Cellulose to Cellobiose Reaction with Competitive Glucose, Cellobiose and Xylose Inhibition

$$r_1 = \frac{k_{1r} E_{1B} R_S S}{1 + \frac{G_2}{K_{1IG_2}} + \frac{G}{K_{1IG}} + \frac{X}{K_{1IX}}} \tag{0.1}$$

Cellulose to Glucose Reaction with Competitive Glucose, Cellobiose and Xylose Inhibition

$$r_2 = \frac{k_{2r} \left(E_{1B} + E_{2B}\right) R_S S}{1 + \frac{G_2}{K_{2IG_2}} + \frac{G}{K_{2IG}} + \frac{X}{K_{2IX}}} \tag{0.2}$$

Cellobiose to Glucose Reaction with Competitive Glucose and Xylose Inhibition

$$r_3 = \frac{k_{3r} E_{2F} G_2}{K_{3M}\left(1 + \frac{G}{K_{3IG}} + \frac{X}{K_{3IX}}\right) + G_2} \tag{0.3}$$

Mass Balances

Cellulose $$\frac{dS}{dt} = -r_1 - r_2 \tag{0.4}$$

Cellobiose $$\frac{dG_2}{dt} = 1.056 r_1 - r_3 \tag{0.5}$$

Glucose $$\frac{dG}{dt} = 1.111 r_2 + 1.053 r_3 \tag{0.6}$$

Enzyme $$E_{Ti} = E_{Fi} + E_{Bi} \tag{0.7}$$

Enzyme Adsorption

Lagmuir isotherm $$E_B = \frac{E_{\max} K_{ad} E_F S}{K_{ad} + E_F} \tag{0.8}$$

Temperature Dependence

Arrhenius equation $$k_{ir(T2)} = k_{ir(T1)} e^{-\frac{E_{ai}}{R}\left(\frac{1}{T1} - \frac{1}{T2}\right)} \tag{0.9}$$

Nomenclature

E_a activation energy (cal/mole)
E_T total enzyme concentration (g/kg)
E_B bound enzyme concentration (g/kg)
E_F free enzyme concentration (g/kg)
E_{1B} bound concentration of CBH & EG (g/kg)
E_{2B} bound concentration of β-glucosidase (g/kg)
E_{2F} concentration of β-glucosidase in solution (g/kg)
E_{max} maximum mass of enzyme that can adsorb per unit mass substrate (g protein/g cellulose)
G glucose concentration (g/kg)
G_2 cellobiose concentration (g/kg)
K_{ad} dissociation constant for enzyme adsorption/desorption (g protein/g cellulose)
k_{ir} reaction rate constants (kg/mg hr)
K_{iIG} inhibition constants for glucose (g/kg)
K_{iIG2} inhibition constants for cellobiose (g/kg)
K_{iIX} inhibition constants for cellobiose (g/kg)
K_{3M} substrate (cellobiose) saturation constant (g/kg)
R universal gas constant (cal/mole.°K)
R_s substrate reactivity

S substrate concentration (g/kg)
T temperature (°K)

The proposed model differs from previous models in distinguishing between the adsorption of β-glucosidase and that of CBH and EG enzymes and representing their adsorption via a Lagmuir-type isotherm. This more structured formulation should permit the model to better describe the kinetics of cellulase preparations having different proportions of these components. Beyond this, the model incorporates potential inhibition by xylose, a prominent sugar in hydrolyzates of dilute-acid pretreated biomass; inhibition of cellulases by xylose has not been considered previously.

Model Validation

Model parameters were estimated from experimental data generated at 45°C using dilute-acid pretreated corn stover (PCS) as the substrate and CPN, a reference cellulase preparation (Table 2). These parameters were used in model validation.

The kinetic model for cellulose saccharification needs to be validated using datasets not used to estimate model parameters. The model was used to simulate cellulose hydrolysis performance at various levels of background glucose, cellobiose, and xylose different than those used in parameter estimation. Validation tests show that the model does a sound job of predicting hydrolysis behavior at high initial glucose, xylose, and cellobiose concentrations (Figure 2 through Figure 4). Thus, inhibition by the three sugars is well captured by the model. Xylose

inhibition as predicted by the model is authentic in the sense that the 24-h hydrolysis efficiencies at 0 and 50 g/kg background xylose are 53% vs. 46%, respectively, suggesting significant xylose inhibition (this was based on a single experiment). The high cellobiose concentration used is only of academic interest since cellobiose concentrations typically peak at much lower levels (10-12 g/L); cellobiose concentrations are sensitive to the β-glucosidase content of a given enzyme preparation.

Table 2. Estimated model parameters

Parameter	Value
Independently Established Parameters	
$K_{ad\text{-}EG/CBH}$ (g protein/g substrate)	0.4
$K_{ad\text{-}\beta\text{-}glucosidase}$ (g protein/g substrate)	0.1
$E_{max\text{-}EG/CBH}$ (g protein/g substrate)	0.06
$E_{max\text{-}\beta\text{-}glucosidase}$ (g protein/g substrate)	0.01
E_a (cal/mole)	-5540
R_s	$\alpha S/S_0$, α=1
Parameters Obtained by Regression of Saccharification Data	
k_{1r} (g/mg hr)	22.3
K_{1IG2} (g/kg)	0.015
K_{1IG} (g/kg)	0.1
K_{1IX} (g/kg)	0.1
k_{2r} (g/mg hr)	7.18
K_{2IG2} (g/kg)	132.0
K_{2IG} (g/kg)	0.04
K_{2IX} (g/kg)	0.2
k_{3r} (hr^{-1})	285.5
K_{3M} (g/kg)	24.3
K_{3IG} (g/kg)	3.9
K_{3IX} (g/kg)	201.0

The model was also used to predict hydrolytic performance at different temperatures. Figure 5 compares experimental glucose concentrations to model predictions at three temperatures. The model predicts hydrolysis behavior reasonably well for 40° and 50°C, but falters at 55°C. This is explained by the fact that the temperature optimum for the CPN enzyme is near 50°C, and the enzyme is slowly inactivated at higher temperatures. Therefore, this aspect of modeling the system needs more work. However, more thermostable second-generation enzymes need to be available to model relevant enzyme inactivation.

Future Model Refinement

The model performed well in predicting cellulose hydrolysis trends at experimental conditions both inside and outside the design space used for parameters estimation, but it could be improved

upon by incorporating the phenomenon of enzyme inactivation and differential hydrolysis potential.

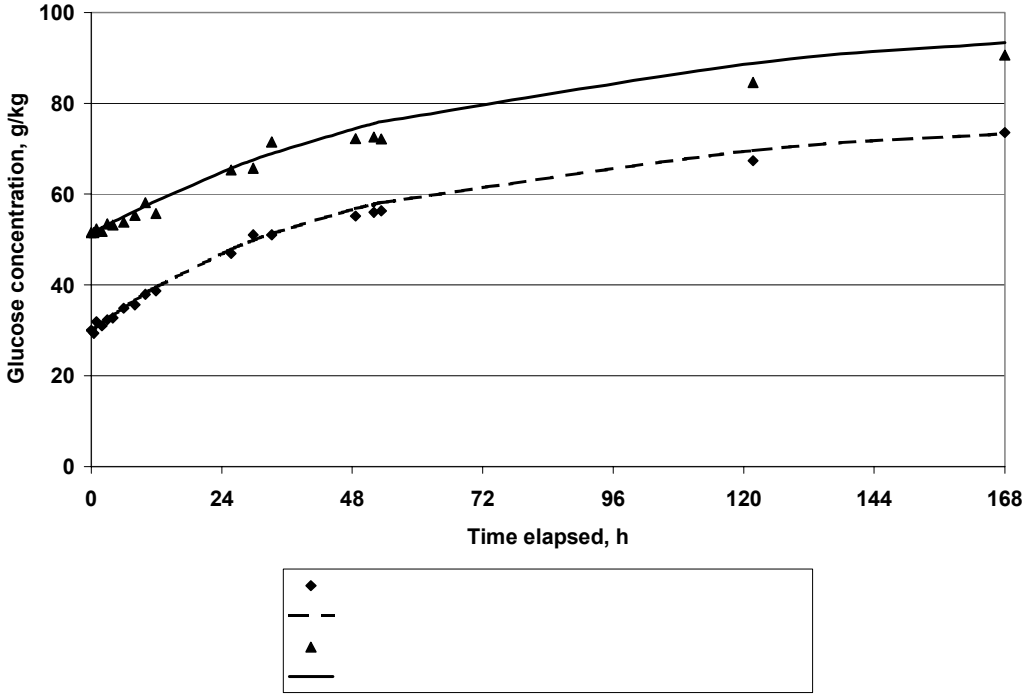

Figure 2. Kinetic model validation. Enzymatic cellulose hydrolysis was conducted in the shake-flask system using 10% w/w corn stover solids with initial background glucose of 30 or 50 g/kg.

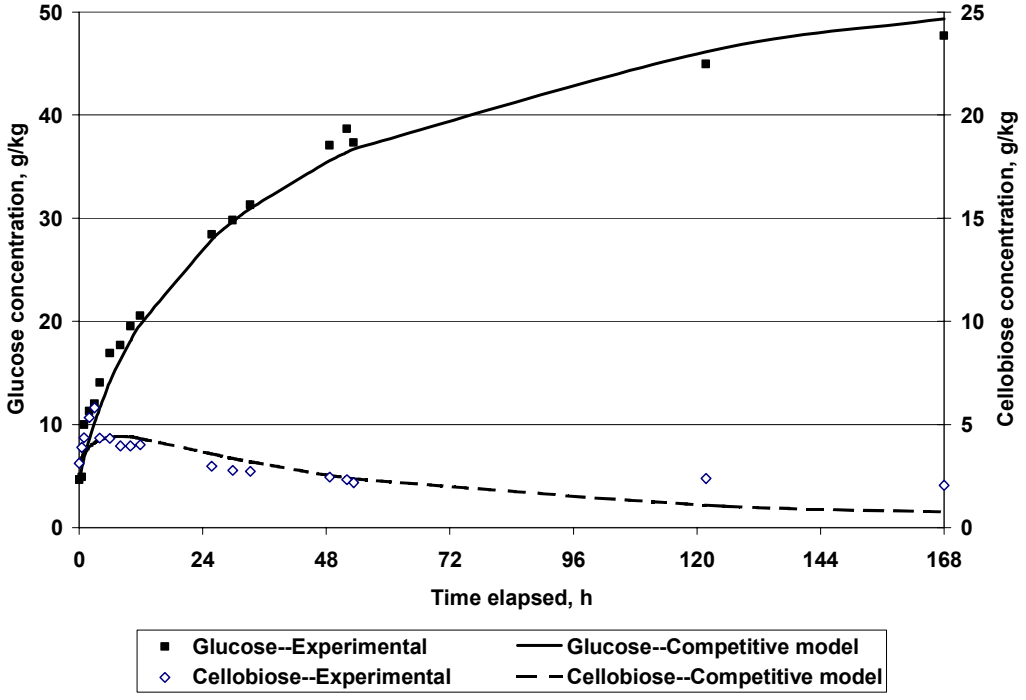

Figure 3. Kinetic model validation. Enzymatic cellulose hydrolysis was conducted in the shake-flask system using 10% w/w corn stover solids with initial background xylose of 50 g/kg.

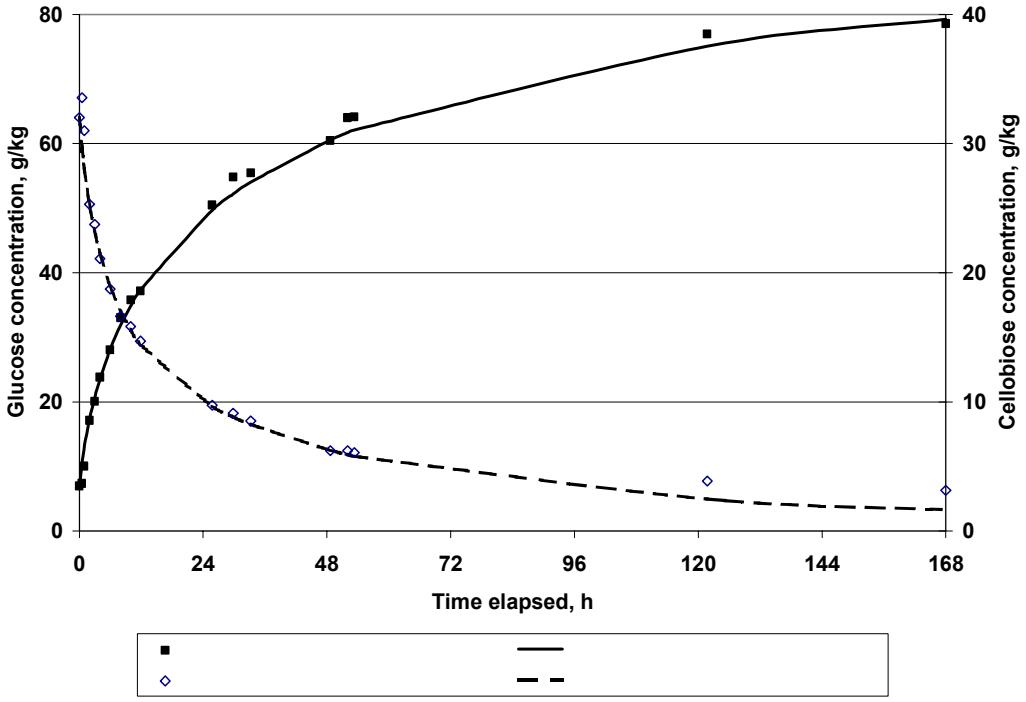

Figure 4. Kinetic model validation. Enzymatic cellulose hydrolysis was conducted in the shake-flask system using 10% w/w corn stover solids with initial background cellobiose of 30 g/kg.

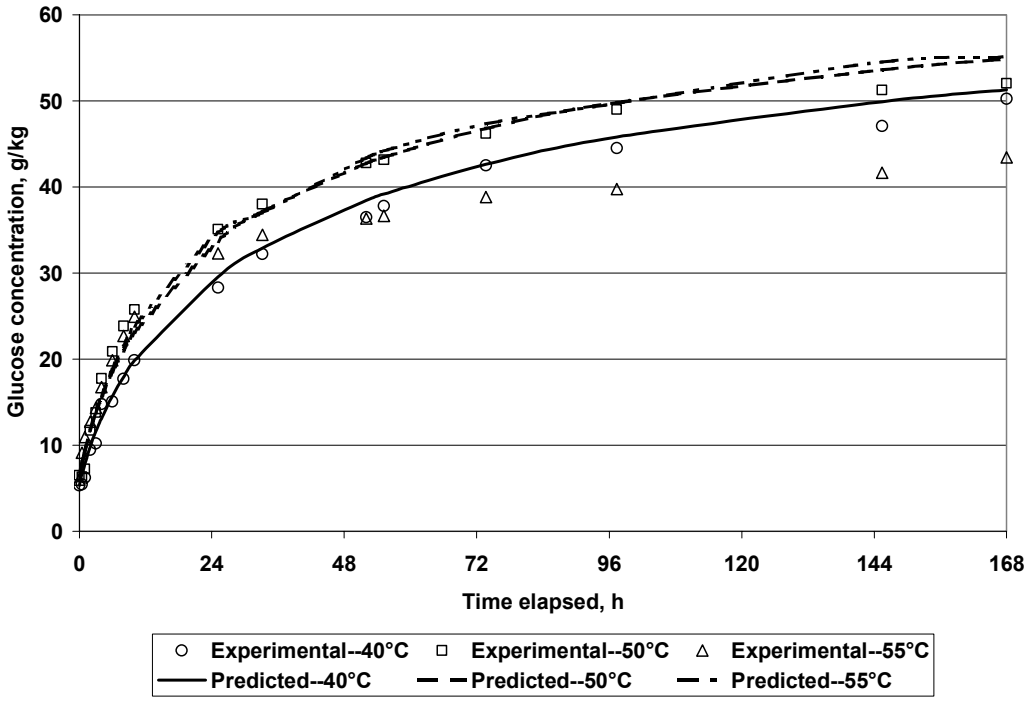

Figure 5. Kinetic model validation. Enzymatic cellulose hydrolysis was conducted in the shake-flask system using 10% w/w corn stover solids at temperatures of 40°C, 50°C, and 55°C.

Corn stover that has been dilute-acid pretreated at different conditions, particularly at different severities, exhibits different susceptibilities to enzymatic digestion. The same is true for corn stover lots containing various amounts of glucan and xylan. This suggests that model parameters developed using a particular batch of pretreated corn stover feedstock may not be sufficient for predicting enzymatic hydrolysis of other batches of pretreated feedstock, i.e., feedstocks of different origin or produced at different pretreatment conditions. An additional parameter to describe feedstock hydrolysis capacity (or enzymatic digestibility potential) can be introduced in the model to accommodate this issue. For example, the extent of cellulose hydrolyzed under defined conditions, as shown below, could be used to account for variable feedstock hydrolysis capacity.

$$\beta = \frac{\text{Maximal hydrolysis yield with new feedstock}}{\text{Maximal hydrolysis yield with reference feedstock}} \tag{0.10}$$

As this work was conducted with a single feedstock, β assumes a value of unity. The substrate reactivity term can be revised to incorporate β as follows:

$$R_S = \beta \frac{S}{S_0} \tag{0.11}$$

Enzyme inactivation as well needs to be incorporated into the model because the enzyme gradually inactivates during prolonged exposure to relatively high temperatures. Enzyme inactivation is related to the duration of exposure to the temperature of hydrolysis milieu, and effective enzyme activity may be suitably expressed as some function of elapsed time. A revised model incorporating these features can be used for *in silico* process optimization.

Potential Correlation between Enzyme Adsorption and Hydrolytic Performance

This study compared two similarly pretreated corn stover samples from different corn stover (Lots 1 and 2) in terms of hydrolytic performance and enzyme adsorption. Based on their SSF performance, they were deemed as least, moderately, or most reactive (Table 3). In addition, the raw feedstock was also subjected to enzyme adsorption studies.

Table 3. Description of corn stover samples

Corn-stover sample ID	Feedstock	Glucan content, % (dry wt)
BMAP 2000	Untreated, Lot 1	36.9
BMAP 112901	Untreated, Lot 2	38.9
P011219CS #1	Most reactive	59.2
P010516CS #3	Least reactive	52.8
P001220CS #4	Moderately reactive	57.9

All the corn stover samples were used as substrates in enzyme adsorption studies, and the three pretreated samples were subjected to saccharification as well. CPN cellulase served as the enzyme source. After 2 h of equilibration all samples were centrifuged immediately. The supernatants were filtered and analyzed for total protein using the Coomassie blue assay. Residual substrate concentration was estimated by measuring the glucose and cellobiose sugars via HPLC. The enzyme adsorption phenomenon was assumed to follow the Langmuir isotherm shown below.

$$E_B = \frac{E_{\max} K_{ad} E_F S}{K_{ad} + E_F}$$

Where,

E_B bound enzyme concentration (g/kg)
E_F free enzyme concentration (g/kg)
E_{max} maximum mass of enzyme that can adsorb onto a unit mass of substrate (g protein/g cellulose)
K_{ad} dissociation constant for the enzyme adsorption/desorption reaction (g protein/g cellulose)
S substrate concentration (g/kg)

Cellulose conversion during saccharification of the pretreated corn stover samples is shown in Figure 6; these profiles are similar to those observed during SSF in that the relative digestibility of these samples is confirmed. As expected, the most reactive material achieved the highest cellulose conversion (87% after 7 d and a combined glucose and cellobiose concentration near 70 g/L). Surprisingly, the performance of the moderately and least reactive material was very similar. The moderately reactive material achieved slightly greater cellulose conversion after 7 d (63%), but this value is not significantly different from the final value (60%) for the least reactive material.

The experimental data and Langmuir isotherm predictions (based on the Langmuir parameters regressed from experimental data) are depicted in Figure 7, which is a plot of E_b/S, the bound enzyme concentration per unit mass of substrate versus E_f, the free enzyme concentration. The vertical line represents the amount of free enzyme available (E_f=1.0 g/kg) at more process relevant conditions (i.e., 15 FPU/g cellulose). The adsorption behavior displayed is as expected with low enzyme adsorption for the raw feedstocks, then increasing adsorption in order of reactivity, with the highest value observed for the most reactive material. There was essentially no difference in measured adsorption characteristics between the two raw feedstocks.

The most reactive material more readily adsorbed enzyme and achieved greater cellulose conversion during both closed system saccharification and SSF. As expected, the performance levels of the negative controls (i.e., the poorly pretreated, least reactive) and the raw corn stover samples were significantly poorer than for the other two materials. All of these results simply confirm that increasing enzyme adsorption correlates well with higher cellulose conversion. These results, however, also have implications for the development of kinetic and

technoeconomic models that utilize corn stover as a feedstock, since material composition and structure appear to influence cellulose conversion characteristics. The effect on process economics is significant as the higher carbohydrate content feedstock increases the sugar available and enables higher hydrolysis yields, thereby facilitating greater ethanol production. The results suggest that we guide farmers and harvesters to select corn varieties and harvesting methods that produce stover higher in carbohydrate content to positively affect process economics.

Process-Relevant Lignocellulose Saccharification

The current NREL conceptual bioethanol process design, as described by Aden et al. (2002), comprises feedstock handling, pretreatment, saccharification, fermentation, and product recovery as basic unit operations. This design served as a guide in selecting experimental conditions to simulate process-relevant lignocellulose saccharification. Dilute-sulfuric acid pretreatment is the baseline pretreatment considered by Aden et al. (2002). Soluble sugar concentrations in a typical corn stover hydrolyzate resulting from dilute-acid pretreatment at 25% solids are as follows (g/L): cellobiose 1.9, glucose 16.7, xylose 69.2, galactose 6.6, arabinose 11.9, and mannose 5.1. However, pretreated corn stover slurries exiting the pretreatment section undergo washing and consequent dilution and typically contain 20% total solids, 5% of which are soluble sugars. The stream entering saccharification is also envisaged to contain trace levels of oligomers (cellobiose and xylobiose).

The relatively high soluble sugar concentrations in slurry liquor imply that resistance to sugar inhibition will be essential to achieve high cellulose conversion yields in the absence of simultaneous fermentation. Enzymatic saccharification must also overcome inhibition by the non-sugar soluble components in slurry liquor which include potentially inhibitory compounds such as acetic acid, low-molecular weight lignin-derived compounds, furfural—the principal degradation product from pentose sugars—and 5-hydroxymethylfurfural, the principal degradation product from hexose sugars. Finally, if a portion of the overall cellulose conversion process is carried out in SSF mode, then enzymatic hydrolysis must also be resistant to inhibition by ethanol. This is the type of system that enzymatic cellulose hydrolysis will be performed in.

Experimental Results

Previous corn-stover saccharification work has been carried out with washed pretreated corn stover. As discussed above, it is necessary to generate realistic performance data for saccharification and other unit operations comprising an integrated bioethanol process. In this context, we have made some progress in characterizing enzymatic cellulose saccharification under process-relevant conditions and understanding how to configure the overall process to maximize intermediate sugars production. Recently completed exploratory integration work using PCS focused on evaluating hydrolyzate conditioning, different enzyme preparations, and solids loading. Shake flask experiments were carried out using either a reference cellulase preparation (CPN) or a commercial product (Spezyme) manufactured by Genencor at 45°C. Flasks were loaded at insoluble solids levels similar to those anticipated at process-scale operations (10-12.5% w/w dry insoluble solids, corresponding to 6-8% w/w cellulose).

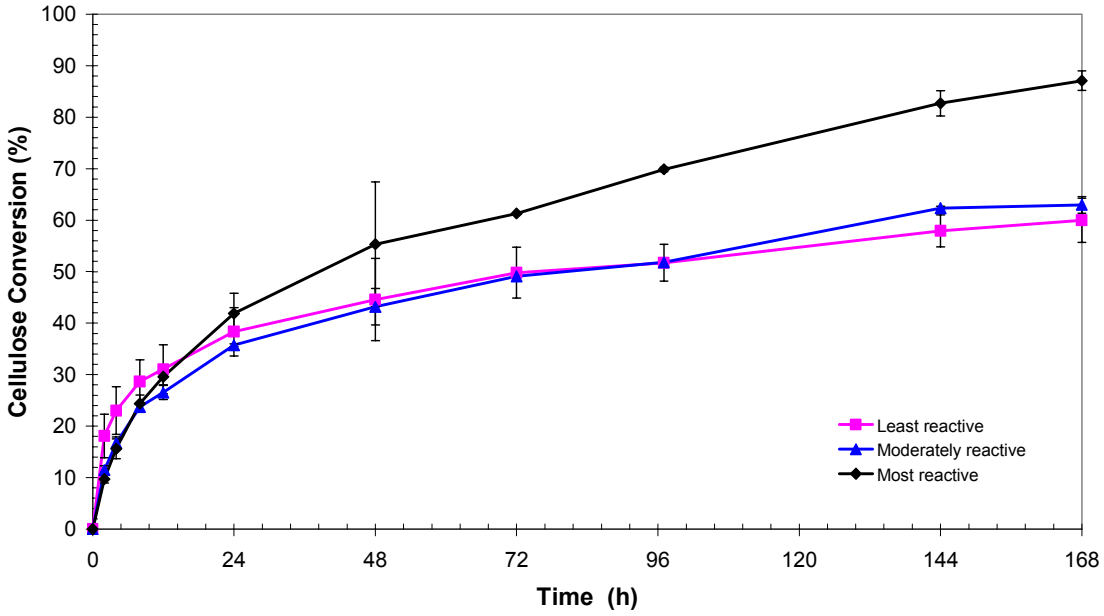

Figure 6. Cellulose conversion data for the three pretreated corn stover samples during saccharification at 45°C.

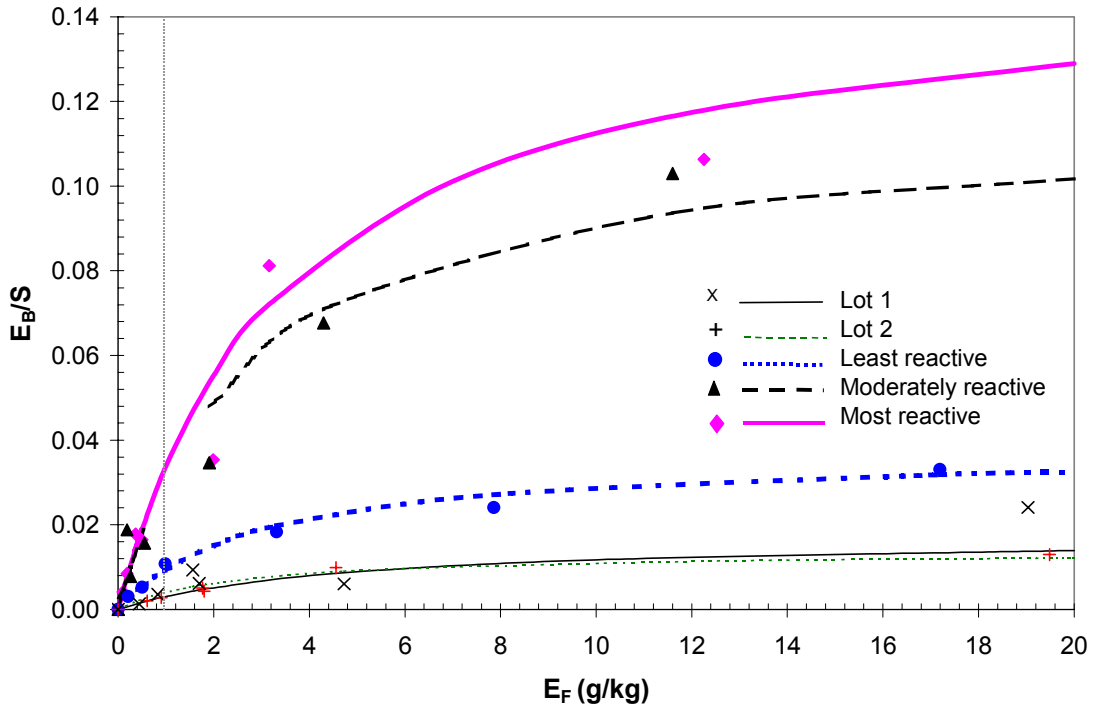

Figure 7. Langmuir adsorption data and predicted values for pretreated and raw corn stover samples.

Figure 8 shows the PCS hydrolysis results using hydrolyzates that were conditioned via overliming or simple neutralization with lime using CPN at 45 mg protein/g cellulose. (These results are suboptimal due to low pH caused by interstitial hydrolyzate, a problem corrected in later experiments.) The difference between their abilities to support PCS hydrolysis is minor. The two methods of conditioning are also comparable as judged from the relative hydrolysis data shown in Figure 9. These results demonstrate that enzymatic saccharification of PCS cellulose proceeds equally well in either neutralized or overlimed hydrolyzate. This portends that there is flexibility as to where overliming is applied in the process as simple neutralization suffices for saccharification. Recombinant strains of *Zymomonas mobilis*, *Saccharomyces cerevisiae*, *E. coli*, and *Klebsiella oxytoca* are candidate ethanologens (Kadam 2001). Depending on their fermentative robustness, the following options are then available 1) neutralization before saccharification only, 2) neutralization before saccharification followed by overliming before fermentation, or 3) overliming before saccharification only.

The effect of hydrolyzate levels on hydrolysis using an enzyme loading of 45 mg protein/g cellulose is shown in Figure 10. Spezyme appears to be less sensitive to hydrolyzate levels than CPN. Different levels of hydrolyzates exhibited fairly similar glucan conversion using Spezyme, whereas CPN's performance dropped significantly at high hydrolyzate levels suggesting susceptibility to increased levels of hydrolyzate toxins. Another observation from this study is that final cellobiose levels rise with increasing hydrolyzate levels, implying a hampering of β-glucosidase performance for both enzyme preparations (Figure 11). Hence, resistance to cellobiose inhibition is a desirable trait for the next generation of cellulases.

To further appraise the Spezyme preparation, higher solids levels and lower enzyme loadings were also tested. As expected, enzyme loading has a positive effect on hydrolysis and the washed substrate does significantly better than the unwashed (Figure 12). Although extensive washing is neither economical nor practical in an industrial process, it is instructive to compare the hydrolytic capacities of washed and unwashed PCS. As illustrated in Figure 12, at higher enzyme loadings the benefits of washing almost disappear. High solids levels seem to reduce the final glucan conversion, indicating that 10% insoluble solids level may be optimal (Figure 13). However, it should be emphasized that the mass-transfer limitations in the shake flasks at higher solids loading may have skewed the results to some extent, and saccharification in a stirred vessel with good mixing is necessary to draw definitive conclusions. Reactor design for effectively mixing non-Newtonian slurries is a challenge in this area.

These studies show that cellulase enzymes can partially hydrolyze high solids loading PCS slurries when significant levels of hemicellulose-derived sugars (predominantly xylose) are present (which is assumed in the base-case process design). A key finding is that initial cellulose conversion rates are appreciable in the presence of relatively high hemicellulose-derived sugar concentrations (≥100 g/L total sugars, ≥60 g/L xylose). As expected, enzymatic cellulose hydrolysis rates decrease with time and extent of conversion, and sugar production essentially halts once the concentration of glucose (and perhaps cellobiose) reaches a critical inhibitory level for a given set of conditions. Results show that under the test conditions glucose concentrations of about 70 g/L can be reached, the maximum concentration to which glucose can accumulate increasing with enzyme loading.

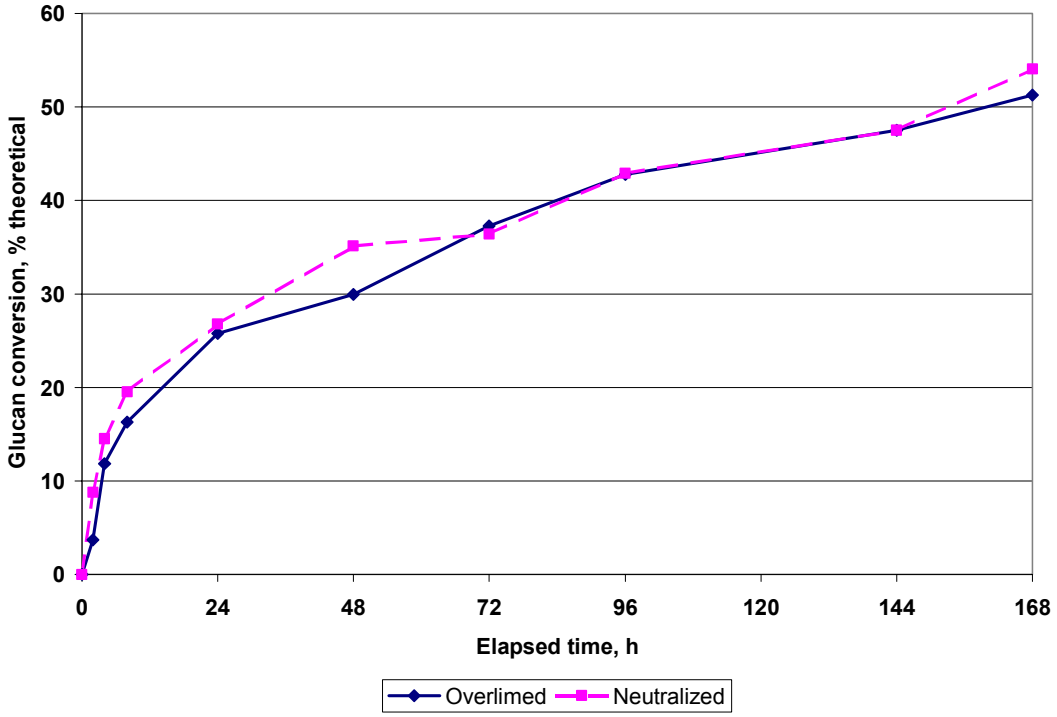

Figure 8. Saccharification of unwashed corn stover in the presence of neutralized or overlimed hydrolyzate (CPN at 45 mg protein/g cellulose).

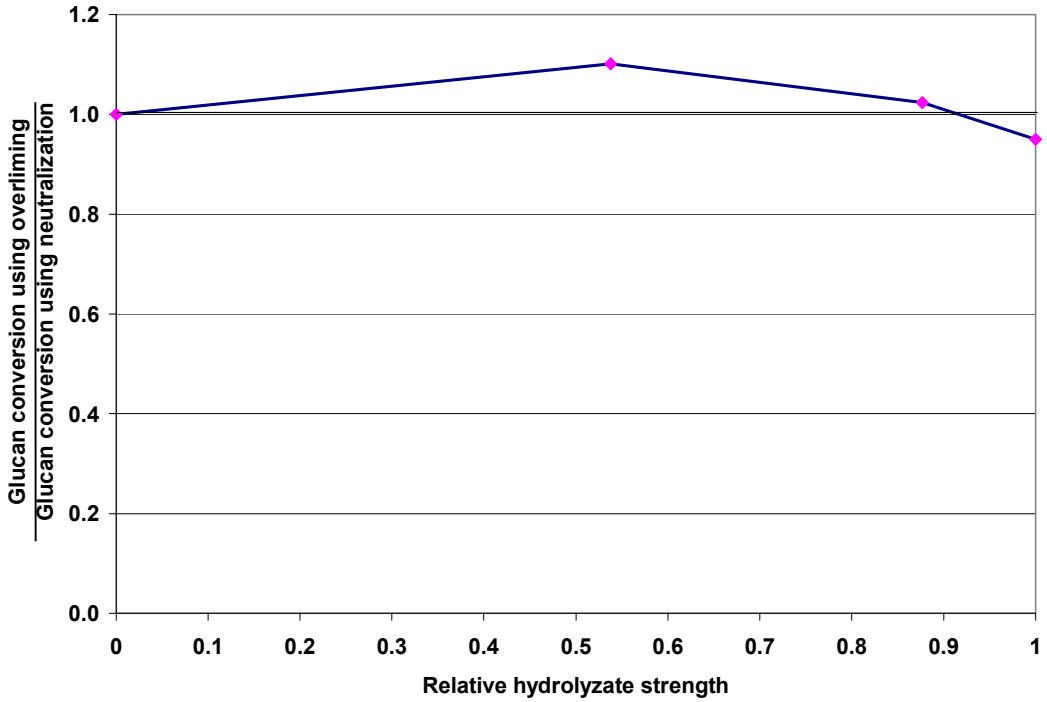

Figure 9. Relative glucan conversion in the presence of neutralized or overlimed hydrolyzate (CPN at 45 mg protein/g cellulose). Hydrolyzate strength is relative to the hydrolyzate from 25%-solids pretreatment.

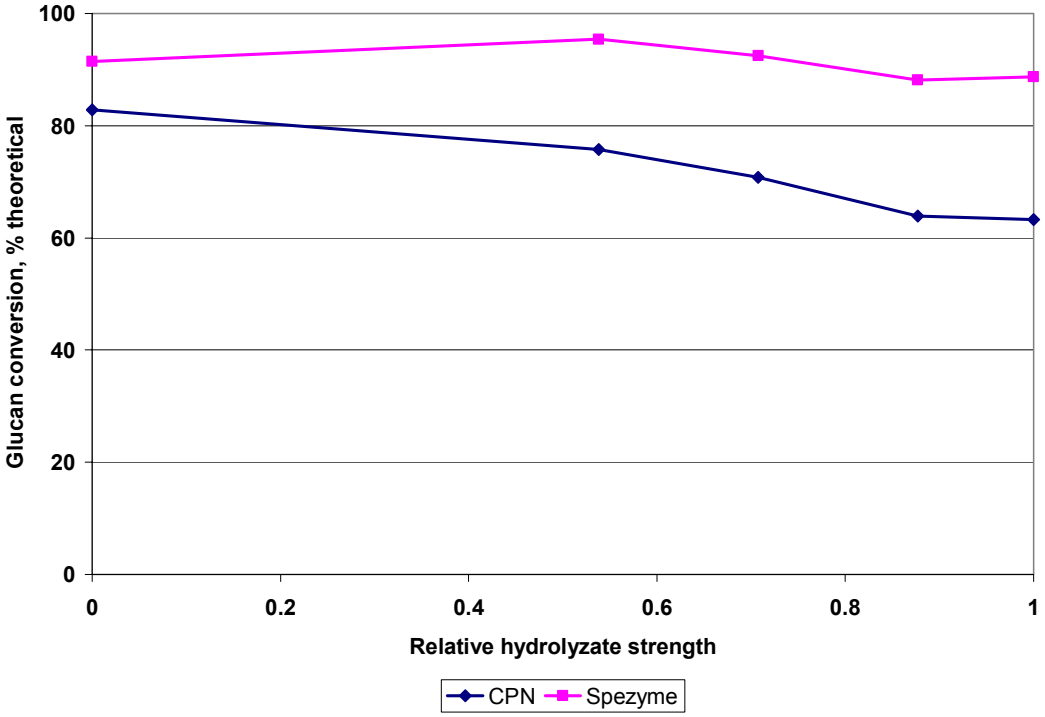

Figure 10. Glucan conversion as a function of neutralized hydrolyzate strength (CPN or Spezyme at 45 mg protein/g cellulose). Hydrolyzate strength is relative to the hydrolyzate from 25%-solids pretreatment.

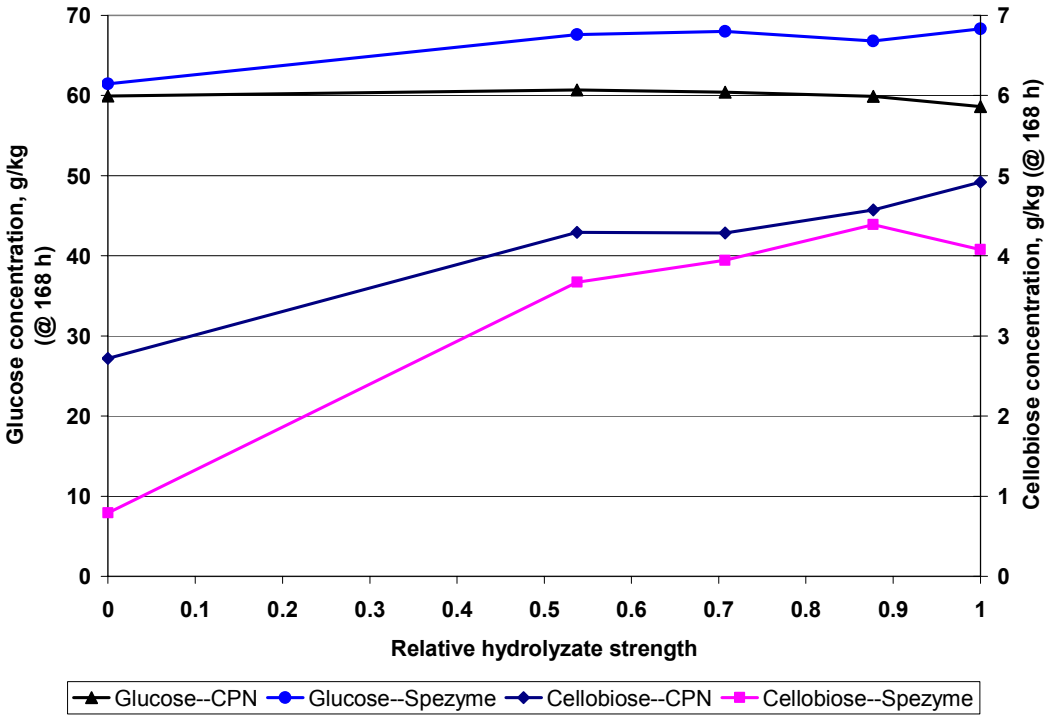

Figure 11. Final sugar concentration as a function of neutralized hydrolyzate strength (CPN or Spezyme at 45 mg protein/g cellulose). Hydrolyzate strength is relative to the hydrolyzate from 25%-solids pretreatment.

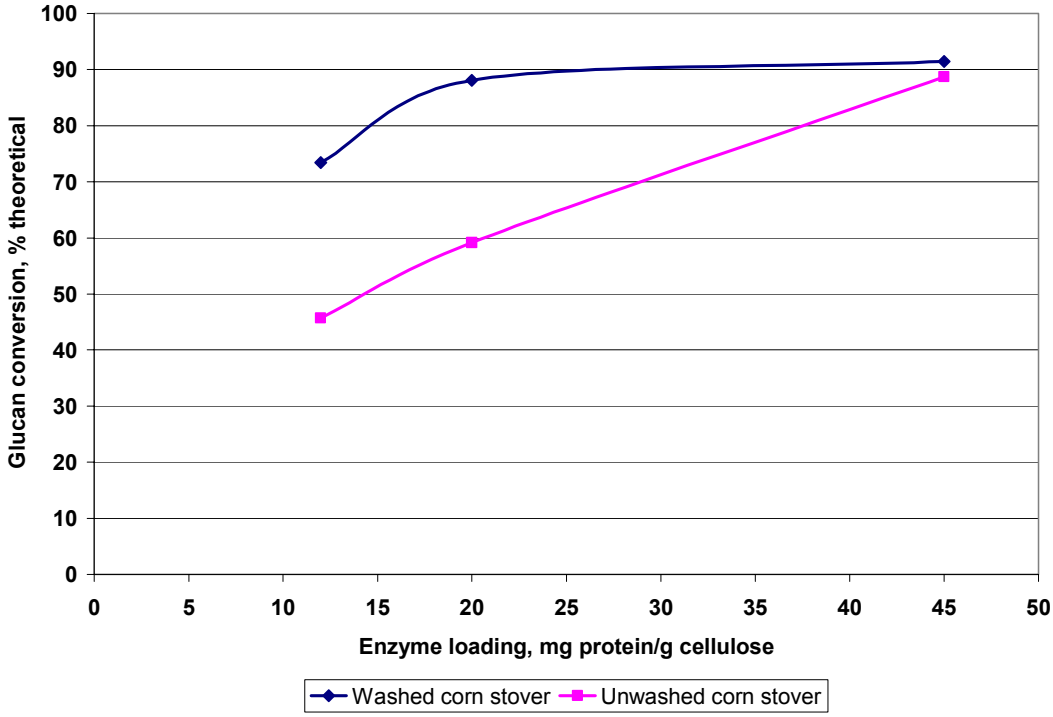

Figure 12. Comparison of the hydrolytic capacities of washed and unwashed corn stover at different enzyme loadings.

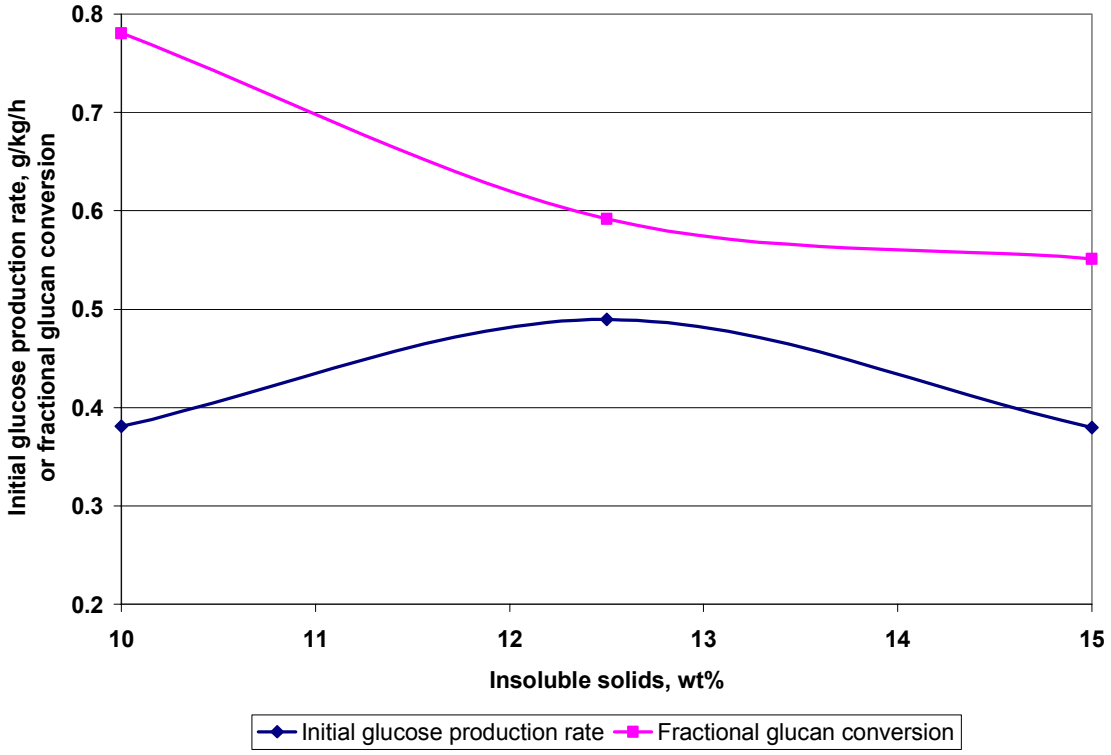

Figure 13. Initial glucose production rate and fractional glucan conversion during saccharification of unwashed corn stover using Spezyme at 20 mg protein/g cellulose.

Recommendations and Future Work

Cellulose Hydrolysis

- Incorporate enzyme inactivation and hydrolysis capacity factor in the kinetic model and validate the new model: To render the model more useful, incorporation of enzyme inactivation and hydrolysis capacity factor into the kinetic model is planned. The feedstock hydrolysis capacity could be used to account for variable feedstock hydrolysis capacities of different corn stover lots containing various amounts of glucan and xylan. Enzyme inactivation also needs to be incorporated because the enzyme gradually inactivates during prolonged exposure to relatively high temperatures. A revised model incorporating these features can be used for *in silico* process optimization.
- Evaluate 2nd generation cellulase enzyme preparations: As Genencor International and Novozymes Biotech Inc. develop improved enzymes these need to be evaluated for their ability to hydrolyze cellulose at process-relevant conditions and assessed for their ability to meet aggressive conversion/rate goals consistent with process engineering model targets. The projected costs from the economic model are based on certain assumptions. It is imperative to assess how the improved enzymes perform in relation to these criteria and to suggest areas for improvements to achieve further cost reductions. This work will build upon process knowledge gained from integrated processing research discussed below.

Integrated Processing Research

- Preliminary process integration research depends on effective hydrolysate conditioning so that relevant performance information can be obtained. Conditioning studies, while not necessarily striving to optimize the process, will explore process-relevant conditioning methods as well as carbon and other component mass balances (e.g., Ca and S) to obtained information to improve process modeling.
- Engineering data for separation processes, in particular, separations involving hydrolysate and fermentation residue are needed to improve process modeling. We propose to use our capabilities for generating large quantities of these materials to develop the necessary data by coordinating our efforts with the process engineering team and their engineering subcontracts as well as using in-house separation equipment (Pnuempress) that is the basis of our recent process designs.
- The fermentation strategy (SSF, SHF, or HHF) is highly dependent upon the microorganism/enzyme system used to produce the bioproduct. Additionally, only commercial track projects will select products, therefore it is premature for this project to invest significant resources investigating fermentation strategies. However, we propose to use model microorganisms (e.g, glucose fermenting yeast and/or recombinant bacteria) to integrated saccharification and fermentation in order to study the factors influencing cellulose hydrolysis as well as to develop the tools and method to improve carbon and mass balance closure for these unit operations.

- Explore reactor designs for effectively mixing corn stover slurries: Working at relatively high solids levels poses some mixing and hydrodynamic problems. Studying the rheological properties of corn stover slurries and exploring reactor designs for effective mixing will advance the general knowledge about the overall process.

Success with the above work would imply transfer of the knowledge and capabilities to commercial track projects. Additional work envisioned for a research track project is discussed below.

- Demonstrate "robustness" under industrially relevant conditions: Operating a given system at the bench-scale (or mini-pilot scale) under industrially relevant conditions and fully integrated for a length of time would be necessary to build a database for process verification. However, this is a lack of experimental systems to achieve this objective.
- Demonstrate integrated process performance at a large enough scale: This would be needed for a process guaranty and preliminary process design. Operational scale and scope will be defined by industrial partner/entity and engineering contractors. NREL and other facilities have some capabilities in this area, but additional requirement would depend on the process configuration.

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